Non-viral vectors for gene-based therapy

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Abstract | Gene-based therapy is the intentional modulation of gene expression in specific cells to treat pathological conditions. This modulation is accomplished by introducing exogenous nucleic acids such as DNA, mRNA, small interfering RNA (siRNA), microRNA (miRNA) or antisense oligonucleotides. Given the large size and the negative charge of these macromolecules, their delivery is typically mediated by carriers or vectors. In this Review, we introduce the biological barriers to gene delivery *in vivo* and discuss recent advances in material sciences, nanotechnology and nucleic acid chemistry that have yielded promising non-viral delivery systems, some of which are currently undergoing testing in clinical trials. The diversity of these systems highlights the recent progress of gene-based therapy using non-viral approaches.

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Almost any sequence in the genome and every coding and non-coding RNA in the transcriptome has the potential to be modulated for therapeutic purposes through the introduction of exogenous nucleic acids into a cell. Over the past two decades, the clinical application of gene-based therapy for treating or preventing a wide range of diseases has been investigated[1]. However, success in clinical trials has been limited owing to numerous technical barriers.

A fundamental engineering challenge of gene-based therapy is the development of safe and effective delivery vectors. Both viral vectors (reviewed in REFS 2,3) and non-viral vectors are used for systemic delivery in clinical trials. In fact, ~70% of gene therapy clinical trials carried out so far have used modified viruses such as retroviruses, lentiviruses, adenoviruses and adeno-associated viruses (AAVs) to deliver genes. Although they have substantially advanced the field of gene therapy, several limitations are associated with viral vectors, including carcinogenesis[4], immunogenicity[5], broad tropism[6], limited DNA packaging capacity[7] and difficulty of vector production[8]. Non-viral gene therapy has the potential to address many of these limitations, particularly with respect to safety. For example, synthetic 'vehicles' tend to have lower immunogenicity than viral vectors, and patients do not have pre-existing immunity as is the case with some viral systems[9,10]. Non-viral vectors also have the potential to deliver larger genetic payloads and are typically easier to synthesize than viral vectors[9,10]. A diverse collection of synthetic vectors has been developed to bring therapeutic nucleic acids to their sites of action. Nevertheless, few of these vectors have so far been developed clinically owing to their low delivery efficiency relative to viral vectors[11]. Whereas viruses have evolved to deliver their genomes efficiently to mammalian cells, most synthetic vectors are unable to effectively transport their payloads past the multiple barriers that confront them.

This drawback may be about to change owing to developments in material sciences, which have yielded new polymers and lipids as delivery vectors[9,12–15], as well as owing to the rapid progress of nanotechnology, which has enabled a better understanding of nanosized materials for gene delivery[16,17]. Moreover, recent advances in nucleic acid chemistry have improved potency and stability while reducing the immunogenicity of RNA molecules[18–21].

In this Review, we summarize the barriers of and challenges to systemic delivery of nucleic acids, and discuss how non-viral vectors can overcome obstacles in the *in vivo* delivery of DNA, mRNA, small interfering RNA (siRNA) and microRNA (miRNA) mimics. In addition, we highlight some clinical trials that indicate the value of non-viral vectors in gene-based therapy. Finally, we briefly comment on the potential use of non-viral vectors for systemic delivery of zinc-finger proteins (ZFPs), transcription activator-like effectors (TALEs) and CRISPR–Cas (clustered regularly interspaced short palindromic repeat–CRISPR-associated) systems, which have emerged as the next generation of tools to achieve precise gene editing with minimal adverse effects.

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In vivo DNA delivery

According to the American Society of Gene & Cell Therapy, DNA therapy remains a strictly experimental approach in the United States, and no gene therapeutics have so far been approved by the US Food and Drug Administration (FDA) despite many clinical trials worldwide. Nevertheless, recent clinical progress, including the approval of the AAV-based gene therapeutic Glybera for use in Europe, has marked a resurgence of optimism for a biomedical field burdened by a tumultuous history. Many non-viral systems have been developed for therapeutic DNA delivery, including the injection of naked DNA alone or in combination with physical methods[22–26] such as gene gun, electroporation, hydrodynamic delivery, sonoporation and magnetofection. These techniques are generally less applicable to systemic gene delivery in humans than in small animals such as mice; hence, a range of synthetic delivery vectors has also been developed, including lipids and liposomes[10,27], polymers (linear and branched polymers, dendrimers and polysaccharides)[9,28,29], polymersomes[30], cell-penetrating peptides[31] and inorganic nanoparticles[32].

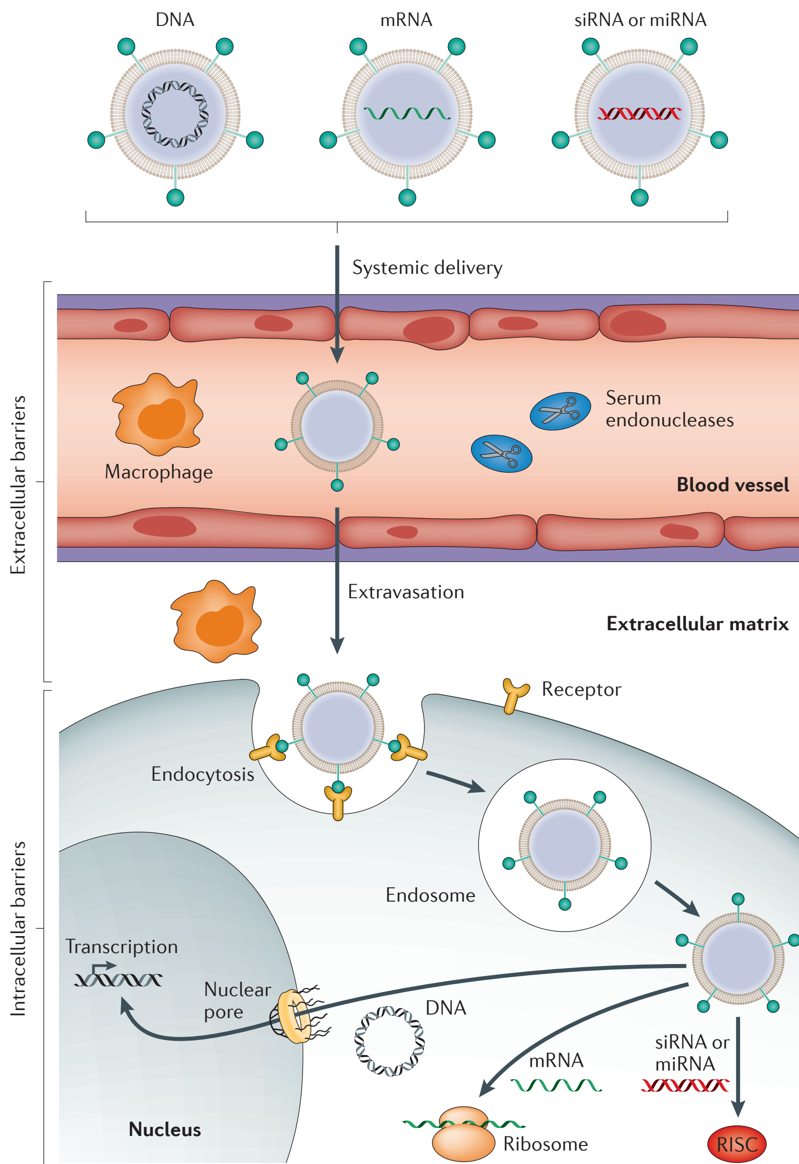

Figure 1 | **Barriers to successful *in vivo* delivery of nucleic acids using non-viral vectors.** Various non-viral vectors can be used to deliver DNA, mRNA and short double-stranded RNA, including small interfering RNA (siRNA) and microRNA (miRNA) mimics. These vectors need to prevent degradation by serum endonucleases and evade immune detection (which could be achieved by chemical modifications of nucleic acids and encapsulation of vectors). They also need to avoid renal clearance from the blood and prevent nonspecific interactions (using polyethylene glycol (PEG) or through specific characteristics of particles). Moreover, these vectors need to extravasate from the bloodstream to reach target tissues (which requires certain characteristics of particles and specific ligands), and mediate cell entry and endosomal escape (by specific ligands and key components of carriers). siRNA and miRNA mimics must be loaded into the RNA-induced silencing complex (RISC), whereas mRNA must bind to the translational machinery. DNA has to be further transported to the nucleus to exert its activity.

Barriers to non-viral DNA delivery. One of the challenges to systemic delivery of DNA therapeutics is the potential degradation of the therapeutic gene by endonucleases in physiological fluids and the extracellular space (FIG. 1). The half-life of plasmid DNA has been estimated to be ten minutes following intravenous injection in mice[33]. For this reason, entrapment of the DNA in a nanoparticulate carrier is desirable both to provide protection from endonuclease degradation and to improve circulation time. For example, zwitterionic lipids have been shown to form a stable structure that envelops DNA and can be used in combination with other formulations to enhance gene delivery *in vivo*[27,34,35]. In addition, polyplexes are spherical or doughnut-shaped nanoparticulate complexes formed from the condensation of negatively charged plasmid DNA by cationic polymers[9]. Similarly, mixtures of cationic lipids, neutral lipids and DNA can spontaneously assemble into lipoplexes or liposomes, in which DNA is entrapped within lamellar or inverted hexagonal arrangements of lipid bilayers[36]. However, at high salt concentrations, electrostatic repulsion between the positively charged complexes is reduced, and they are therefore prone to colloidal instability and aggregation in physiological fluids[37]. In particular, aggregation of nanoparticles in the blood as a result of either colloidal instability or interaction with blood components (such as serum proteins and erythrocytes) can inhibit localization to the desired tissues, induce rapid clearance by circulating macrophages and even cause embolism in lung capillaries[38].

The challenges of selective accumulation at the tissue of interest, cellular internalization and endosomal escape are common to the delivery of all nucleic acid therapeutics. However, DNA delivery must also provide transport into the nucleus to allow access to the transcriptional machinery (FIG. 1). It was reported nearly 30 years ago that direct microinjection of plasmid DNA that encoded thymidine kinase into the nuclei of thymidine kinase-deficient cells resulted in expression of the kinase in 50–100% of the nuclei, as detected by the incorporation of ^{3}H-thymidine into DNA following autoradiographic analyses[39]. However, in >1,000 cells that received cytoplasmic injections of the same plasmid DNA, no thymidine kinase activity was detected. The importance of the

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Retroviruses
Single-stranded RNA viruses that use reverse transcriptase to transcribe their RNA into DNA, which can be integrated into the host genome. The viral DNA can be transcribed, translated and packed into new viruses. Replication-defective retroviruses are commonly used for gene therapy purposes. Most of these retroviruses are only active in dividing cells.

Lentiviruses
A subclass of retroviruses that are active in non-dividing cells. The replication-defective viruses are used both as a research tool to introduce a gene *in vitro* or *in vivo* and for gene therapy. They infect cells with high efficiency and introduce stable expression.

Adenoviruses
DNA viruses that do not integrate into the host genome and that usually do not replicate during cell division. Many of them trigger fast immune responses. For gene therapy purposes, they are usually applied in conditions in which temporary expression of proteins is required.

Adeno-associated viruses
(AAVs). DNA viruses that have very low but measurable genome integration rates and that are used as vectors for gene therapy. They are able to infect both dividing and non-dividing cells with high efficiency and long persistence. Although they have small packing capability, they are preferred for gene therapy owing to their low immunogenicity and low cytotoxicity.

Zinc-finger proteins
(ZFPs). DNA-binding proteins that consist of tandem arrays of zinc-fingers, which are protein structure motifs that contain one or more zinc ions. Engineered zinc-fingers have been shown to recognize three specific base pairs of DNA sequences and can be assembled in tandem to recognize specific nucleic acid sequences. The process of engineering is difficult and requires expertise.

nuclear barrier is further highlighted by the observation that quiescent or slowly dividing cells with intact nuclei are generally more difficult to transfect than cells that divide rapidly and that undergo frequent breakdown of their nuclear envelopes[40]. Both deterministic and stochastic kinetic models of synthetic gene delivery have identified nuclear uptake as a potential rate-limiting step[41,42].

Vector unpacking is generally assumed to be necessary for DNA release and gene expression, but the extent to which dissociation affects gene delivery is unclear. For lipoplexes, it has been proposed that fusion of the cationic lipid with endosomal membrane lipids facilitates not only endosomal escape but also DNA release[43]. Polyplexes have been observed to localize intact to the nucleus, where they presumably undergo dissociation[44,45]. For certain polyplexes, mechanistic studies have reported slow vector unpacking as an explanation for decreased transfection efficiency[46]. Lipoplex-delivered plasmids are more efficiently expressed by nearly tenfold — on the basis of protein expression per plasmid number in the nucleus — than polyplex-delivered plasmids, which might be a potential consequence of incomplete polyplex dissociation in the nucleus[47].

Expression of the transgene and production of the protein of interest are required for function. Plasmids are routinely used as expression vectors in non-viral gene therapy studies owing to their ease of construction and amplification. Moreover, plasmids are episomal and non-integrating, which reduces the risk of insertional mutagenesis compared with viral vectors. The choice of enhancer–promoter combination has a great impact on both the level and the duration of transgene expression. Viral enhancers and promoters derived from cytomegalovirus (CMV), respiratory syncytial virus (RSV) and simian virus 40 (SV40) are frequently used to achieve high levels of expression in a range of mammalian cell and tissue types, but this expression is often transient[48]. Constitutive mammalian promoters, such as the human ubiquitin C (*UBC*) and the eukaryotic translation elongation factor 1 alpha 1 (*EEF1A1*) promoters, have been observed to result in more persistent expression[49]. Tissue-specific promoters — such as the alpha-fetoprotein (*AFP*) enhancer–albumin (*ALB*) promoter, the expression of which is restricted to the liver[50] — offer the possibility of enhanced safety by minimizing off-target transgene expression. Numerous *cis*-acting sequences, including various polyadenylation signals[51], introns[51] and scaffold/matrix attachment regions (S/MARs)[52], have been reported to increase the level and persistence of transgene expression. Moreover, DNA size and topology have been shown to affect gene expression efficiency, and small covalently closed circular plasmids could mediate greater levels of transgene expression than large or linearized constructs[53]. Compact DNA vectors that lack a bacterial backbone (which are known as minicircles) maintain superior levels and duration of gene expression relative to full-length DNA plasmids[54,55]. In an effort to promote longer-term expression, various systems for transgene integration have been developed, including transposition systems based on the recombinases phiC31 (REF. 56), PiggyBac[57] and Sleeping Beauty[58]. However, the safety of these integrating systems with respect to undesired adverse effects as a result of transgene insertion has not yet been established[2].

Lipid-based DNA vectors. Lipid-based vectors are among the most widely used non-viral gene carriers. It was first shown in 1980 that liposomes composed of the phospholipid phosphatidylserine could entrap and deliver SV40 DNA to monkey kidney cells[59]. More efficient transfection was obtained in a later study, which showed that the synthetic cationic lipid DOTMA spontaneously formed small, uniform liposomes that were capable of efficient encapsulation and delivery of DNA to various mammalian cell lines[60]. Cationic lipids such as DOTMA are characterized structurally by three components: a cationic head group, a hydrophobic tail and a linking group between these domains[10]. DOSPA, DOTAP, DMRIE and DC-cholesterol[10] feature particular modifications of these three domains and are examples of cationic lipids that have been used for liposomal gene delivery[43] (FIG. 2a). Neutral lipids, such as the fusogenic phospholipid DOPE or the membrane component cholesterol, have been included in liposomal formulations as 'helper lipids' to enhance transfection activity and nanoparticle stability[27]. Limitations of cationic lipids include low efficacy owing to poor stability and rapid clearance[61], as well as the generation of inflammatory or anti-inflammatory responses[62]. Recently, Allovectin-7, which is a locally administered formulation consisting of DMRIE–DOPE and a DNA plasmid, failed to meet its efficacy end points in a Phase III clinical trial for treatment of advanced metastatic melanoma[63]. Nonetheless, various liposomal formulations continue to be developed clinically, including DOTAP–cholesterol, GAP-DMORIE–DPyPE and GL67A–DOPE–DMPE–polyethylene glycol (PEG) (TABLE 1).

Polymeric DNA vectors. Cationic polymers constitute an alternative class of non-viral DNA vectors and are attractive partly as a result of their immense chemical diversity and their potential for functionalization. Early examples of polymeric DNA vectors are poly(L-lysine) (PLL) and polyethylenimine (PEI). PLL is a homopolypeptide of the basic amino acid lysine (FIG. 2b), and its ability to condense DNA has been known at least since the 1960s (REFS 64,65). Pioneering studies in the late 1980s indicated that PLL conjugated to the asialoorosomucoid glycoprotein could potentially be applied in non-viral liver-targeted gene delivery[66,67]. In general, in the absence of a lysosomal disruption agent such as chloroquine, PLL has fairly poor transfection activity, presumably because at physiological pH its amine groups tend to be positively charged and therefore have low capacity for endosomal buffering and lysis[10]. Moreover, unmodified PLL shows fairly marked *in vitro* cytotoxicity[68]. Numerous modified variants of PLL with enhanced gene delivery properties have been reported[69]. One example includes PLL covered with the hydrophilic polymer PEG, which is designed to minimize nonspecific interaction with serum components and thereby increase circulation time[70,71]. The clinical potential of PEGylated PLL was investigated as a vector to treat cystic fibrosis, and there

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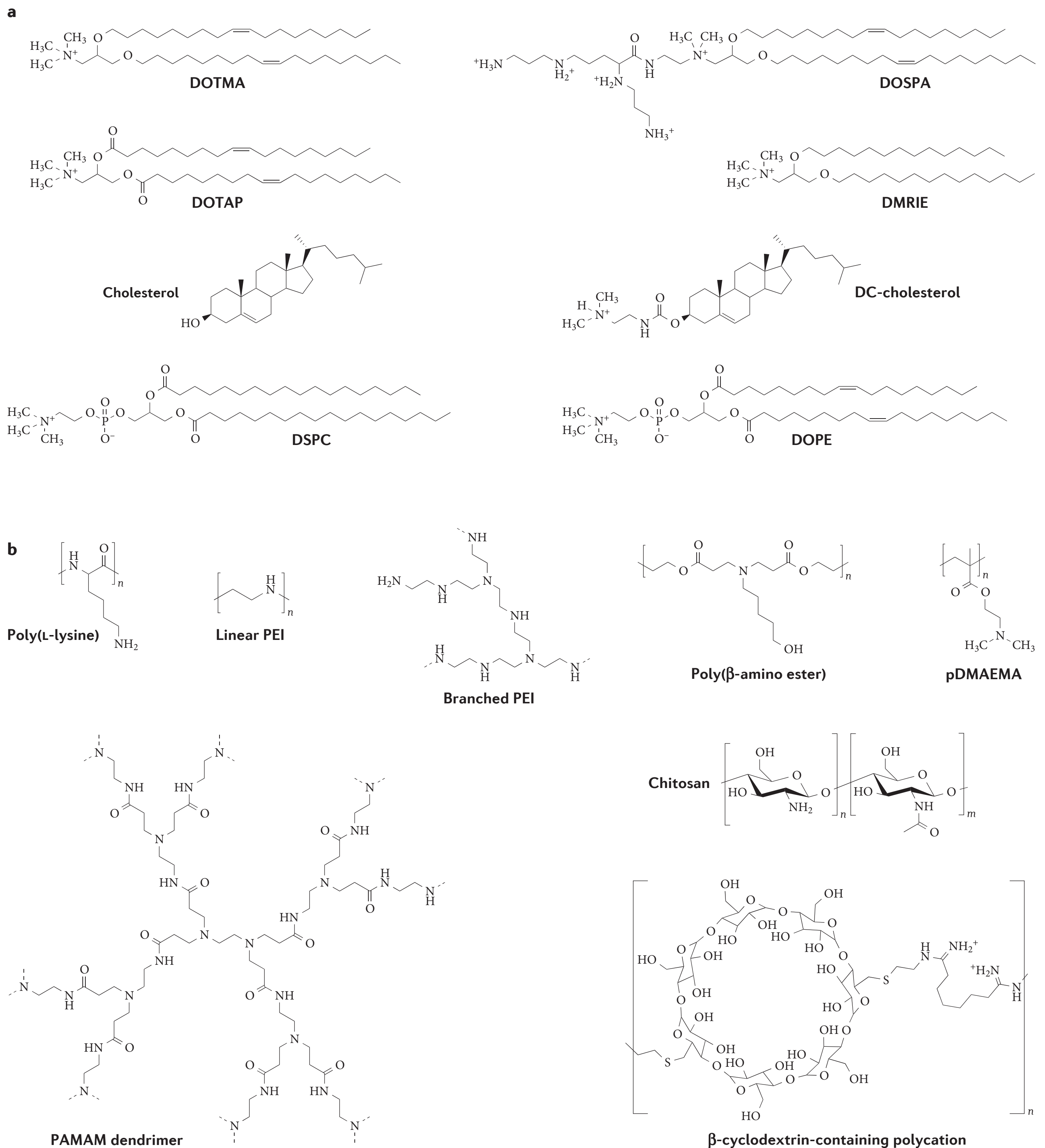

Figure 2 | **Chemical structures of non-viral DNA vectors. a** | Chemical structures of cationic and neutral lipids are shown. Liposomal formulations used for DNA delivery typically include a mixture of a neutral lipid and a cationic lipid. Cationic lipids (such as DOTMA, DOSPA, DOTAP, DMRIE and DC-cholesterol) have an active role in DNA binding and transfection. They are characterized structurally by a cationic head group, a hydrophobic tail and a linker region. Neutral lipids (such as the phospholipids DSPC and DOPE, and the membrane component cholesterol) function as 'helper lipids' to further enhance nanoparticle stability and overall transfection efficacy. **b** | Chemical structures of selected polymeric DNA vectors that are commonly used in gene delivery studies and clinical trials are shown. Poly(L-lysine) and polyethylenimine (PEI) are among the oldest and most commonly used polymeric gene vectors. To improve safety and efficacy, numerous other polymers have been studied for gene delivery, including methacrylate-based polymers such as poly[(2-dimethylamino) ethyl methacrylate] (pDMAEMA), carbohydrate-based polymers such as chitosan and β-cyclodextrin-containing polycations, polyamidoamine (PAMAM) dendrimers and degradable poly(β-amino ester) polymers.

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Table 1 | **Non-viral DNA vectors under clinical evaluation**

Delivery system	Gene therapy drug	Sponsor	Indications	Phase	Status	ClinicalTrials.gov identifier
DOTAP–cholesterol	DOTAP–Chol-fus1	MD Anderson Cancer Center	Non-small-cell lung cancer	I	Completed	NCT00059605
				I/II	Active	NCT01455389
GAP-DMORIE–DPyPE	Tetravalent dengue vaccine	US Army Medical Research and Materiel Command	Dengue disease vaccine	I	Active	NCT01502358
GL67A–DOPE–DMPE–PEG	pGM169/GL67A	Imperial College London	Cystic fibrosis	II	Active	NCT01621867
PEI	BC-819/PEI	BioCancell Ltd.	Bladder cancer	II	Active	NCT00595088
	BC-819	BioCancell Ltd.	Ovarian cancer	I/II	Completed	NCT00826150
	DTA-H19	BioCancell Ltd.	Pancreatic cancer	I/II	Completed	NCT00711997
	SNS01-T	Senesco Technologies, Inc.	Multiple myeloma and B cell lymphoma	I/II	Recruiting	NCT01435720
	CYL-02	University Hospital, Toulouse	Pancreatic ductal adenocarcinoma	I	Completed	NCT01274455
PEG–PEI–cholesterol	EGEN-001	Gynecologic Oncology Group	Ovarian, tubal and peritoneal cancers	I	Recruiting	NCT01489371
				II	Active	NCT01118052
	EGEN-001-301	EGEN, Inc.	Colorectal peritoneal cancer	I/II	Recruiting	NCT01300858
PEI–mannose–dextrose	DermaVir/LC002	Genetic Immunity	HIV vaccine	II	Active	NCT00711230
Poloxamer CRL1005–benzalkonium chloride	ASP0113	Astellas Pharma Inc.	CMV vaccine	III	Recruiting	NCT01877655
				II	Recruiting	NCT01903928
	VCL-CB01	Astellas Pharma Inc.	CMV vaccine	II	Completed	NCT00285259

CMV, cytomegalovirus; PEG, polyethylene glycol; PEI, polyethylenimine.

is a Phase I clinical trial supporting the safety and tolerability of these DNA nanoparticles, as well as some evidence for vector gene transfer[72].

PEI and its variants are among the most studied polymeric materials for gene delivery (FIG. 2b). With a nitrogen atom at every third position along the polymer, PEI has a high charge density at reduced pH values. This attribute of PEI has been postulated to aid in condensation of DNA and endosomal escape[73]. The ability of PEI to promote gene transfection *in vitro* and *in vivo* was first demonstrated in 1995 (REF. 74). Soon after, it was shown that the transfection efficiency and cytotoxicity of PEI strongly depend on its structural properties, especially with respect to molecular weight[75] and the linear versus branched forms[76] (FIG. 2b). In mice, intravenous injection of PEI–DNA polyplexes has been observed to afford gene transfection in the lungs[77], perhaps as a result of the accumulation of nanoparticle aggregates in pulmonary capillaries[78]. Successful intratumoural gene delivery in mice using PEI–DNA polyplexes has been reported[79]. In humans, PEI has been studied for local gene therapy of various cancers (TABLE 1). Nonetheless, as PEI has been known to induce substantial cytotoxicity[80], a range of modifications to PEI has been investigated. Examples include block co-polymers of PEG and PEI for improved stability and biocompatibility[81,82], degradable disulphide-crosslinked PEIs for reduced toxicity[83] and alkylated PEI for increased potency[84,85]. A PEG–PEI–cholesterol lipopolymer is under clinical investigation for immunotherapy of ovarian and colorectal cancers through forced expression of the cytokine interleukin-12 (IL-12) (TABLE 1).

To address issues of efficacy and toxicity associated with PLL and PEI, numerous other polymers are currently being evaluated preclinically for DNA delivery, including poly[(2-dimethylamino) ethyl methacrylate] (pDMAEMA), poly(β-amino ester)s, and various carbohydrate-based polymers and dendrimers[9,10] (FIG. 2b). One vector that has reached clinical development is a formulation that comprises the non-ionic poloxamer CRL1005 and the cationic surfactant benzalkonium chloride. This formulation is being evaluated in a Phase II/III study for a genetic vaccine to prevent CMV infection in patients who are undergoing allogeneic haematopoietic cell transplant (TABLE 1).

In brief, although substantial progress has been made in the field of non-viral gene therapy over the past three decades, DNA-based drugs inherently pose greater delivery and safety challenges than other nucleic acid therapeutics as a result of their large molecular sizes, the difficulty of crossing the nuclear barrier and the risk of mutagenesis. Further clinical progress will be facilitated by additional biological insights into the key rate-determining steps that limit effective delivery and by a more complete understanding of structure–function relationships for DNA delivery materials.

In vivo mRNA delivery

The potential of mRNA for therapeutic protein expression *in vivo* has been investigated as an alternative to DNA-based gene therapy. Although mRNA is less stable than DNA, one of its advantages is reduced immunogenicity. Moreover, there is no potential for mutagenesis

Transcription activator-like effectors
(TALEs). Proteins that were first discovered in *Xanthomonas* spp. bacteria and that bind to promoter sequences in host plants to facilitate infections. They contain a repeat domain of 34 amino acids. Two critical amino acids in each repeat allow targeting of specific DNA bases. TALEs can be engineered in a time-consuming process by assembling repeat domains to recognize specific DNA sequences.

CRISPR–Cas
(Clustered regularly interspaced short palindromic repeat–CRISPR-associated). Defense systems against foreign DNA in bacteria and archaea, in which a short CRISPR RNA (crRNA) is used to guide the Cas nuclease to a specific target DNA sequence. These systems have been optimized to function in mammalian cells with high efficiency. The engineering process to target various DNA sequences is straightforward, and the cost is low.

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Liposomes
Vesicles of various sizes with a lipid bilayer that can encapsulate small molecules or large molecules such as small interfering RNA or DNA. By fusing to the cell membrane, they facilitate delivery of the liposome contents *in vitro* and *in vivo*.

Polymersomes
Vesicles produced using co-polymers, which are made of two or more monomers, to allow both hydrophilic and lipophilic ability. They can encapsulate small molecules, proteins and DNA to form particles of various sizes, and are used for drug delivery systems *in vitro* and *in vivo*.

Cell-penetrating peptides
Small peptides that can translocate across plasma membranes. By covalent or non-covalent binding of these peptides to small molecules, proteins, DNA, small interfering RNA or even nanoparticles, they could facilitate intracellular delivery of various types of molecules.

Scaffold/matrix attachment regions
(S/MARs). Anchor points of the genomic DNA for the chromatin scaffold. They are found at introns or borders of transcription units, where they have an important role in separating these units and regulating gene expression.

PhiC31
A sequence-specific recombinase that mediates recombination between 2 specific 34-bp sequences, which allows insertion of DNA into another DNA molecule or into the genome at specific sites.

PiggyBac
A transposon system composed of a transposon and a transposase, which recognizes transposon-specific sequences on both ends of a vector and integrates the content into different DNA molecules or genome.

Sleeping Beauty
A transposon system reconstructed from DNA copies of salmon. The transposase was engineered to facilitate robust and stable gene transfer.

that results from genomic integration, and mRNA does not require nuclear localization for expression[1,2] (FIG. 1).

Barriers to non-viral mRNA delivery. As mRNA molecules are too large, hydrophilic and negatively charged to diffuse across cell membranes on their own, delivery materials or chemical modifications are generally required to bring therapeutic mRNA to its site of action — namely, to the cytosol of target cells. Systemically administered mRNA delivery systems are subject to a range of challenges to reach their site of action in the body. The ideal mRNA delivery system must protect against serum endonucleases, evade immune detection, prevent nonspecific interactions with proteins or non-target cells, avoid renal clearance, allow extravasation to reach target tissues and promote cell entry (FIG. 1). Methods developed to overcome obstacles for RNA delivery include chemical modification, direct injection and the use of nanoparticles as carriers.

Non-viral mRNA delivery systems. Unmodified mRNA can activate various Toll-like receptors (TLRs) and trigger an increase in cytokine levels and associated toxicity[20,86,87]. The modification of mRNA with a combination of 2-thiouridine and 5-methylcytidine has been shown to reduce immune stimulation through pattern recognition receptors such as TLR3, TLR7, TLR8 and retinoic acid receptor responder protein 3 (RARRES3; also known as RIG-I)[87], whereas the inclusion of pseudouridine in the mRNA (Ψ-mRNA) prevented activation of pattern recognition receptors[18] and 2′-5′-oligoadenylate synthetase[19]. These modifications can also stabilize the mRNA against cleavage and ultimately improve expression rates[20].

The serum protein erythropoietin (EPO) has been commonly used to test mRNA delivery owing to its potential as a therapeutic target and the ability to detect its expression in a non-invasive manner. EPO-encoding mRNA has been administered to mice using intraperitoneal, subcutaneous and intramuscular injections either naked or as a complex with a non-liposomal cationic polymer–lipid formulation[20,87]. Serum EPO levels generally peak several hours after mRNA administration but may be detected for more than a week depending on the dose and method of delivery[20,87].

Successful intranasal delivery and systemic delivery have been observed in mice using luciferase mRNA *in vivo*. Intranasal delivery was reported by complexing the mRNA either to the transfection reagent Stemfect or to a hydrophobic poly(β-amino ester) that had been coated with a positively charged lipid layer[88,89]. The mRNA complexed to Stemfect could also express luciferase in the spleen when injected intravenously[89].

Several studies have already begun to examine the therapeutic potential of mRNA. A direct intramyocardial injection of vascular endothelial growth factor A (VEGF-A)-encoding modified mRNA (modRNA) complexed with RNAiMAX was reported to result in reduced infarct size and apoptotic cell frequency in mice that had a myocardial infarction[86]. Whereas the control group showed <20% survival by day 180, the mice treated with VEGF-A-encoding modRNA had ~60% survival at the end of 1 year[86]. In another study, intranasal delivery of modified mRNA was used to treat congenital deficiency in pulmonary surfactant-associated protein B (PSPB) in mice. Untreated PSPB-deficient mice demonstrated 0% survival by day 5, whereas mice given repeated aerosolized administrations of modified PSPB-encoding mRNA showed >80% survival by day 30 (REF. 87).

Taken together, although the idea of mRNA delivery for gene-based therapy seems to be straightforward, mRNA therapeutics have not yet been fully developed and tested in clinical trials owing to immune responses, instability and delivery barriers. However, encouraged by recent progress in chemical modifications of mRNA and delivery vectors, several biotechnology companies that focus on the development of mRNA therapeutics have been founded and expanded.

In vivo siRNA delivery

Synthetic siRNAs are chemically synthesized double-stranded RNAs with structures that mimic the cleavage products of the enzyme Dicer. They are typically 19–21 bp in length with 2-nucleotide single-stranded overhangs at their 3′ ends. Upon introduction into the cytoplasm, synthetic siRNAs are incorporated into the RNA interference (RNAi) machinery in the same way as endogenous small RNAs. The great therapeutic potential of siRNA is a result of its ability to silence nearly any targeted gene after introduction into cells.

Challenges of* in vivo *siRNA delivery. The biggest obstacle to the therapeutic use of siRNA is the need for *in vivo* delivery. The delivery barriers for siRNA closely resemble the obstacles for mRNA delivery. In addition, siRNA requires integration into the RNAi machinery[61,90] (FIG. 1).

When administered intravenously, unmodified RNA can be cleaved by serum endonucleases and cause activation of innate immunity[91–93]. Both nuclease susceptibility and immunogenicity can be prevented by modifying the chemical structure of the siRNA strands. Common chemical modifications include replacement of the 2′ OH group of ribose with -*O*-methyl or 2′ fluoro groups, incorporation of locked or unlocked nucleic acids and substitution of phosphorothioate linkages in place of phosphodiester bonds[21]. Careful incorporation of these chemical modifications, along with judicious selection of the siRNA sequence, can prevent both degradation by endonucleases[91,92] and recognition by the innate immune system[94]. Another approach to shield siRNA from degradation and immune recognition is to encapsulate it inside nanoparticles[95].

Interaction between delivery systems and various serum components can influence delivery in diverse ways. Some systems rely on interaction with serum proteins to permit their uptake by target cells[96,97] (the mechanisms of which are summarized in REF. 16). For example, some siRNA conjugates and liposomal delivery particles interact with serum lipoproteins that facilitate their specific delivery into hepatocytes[96,97]. However, particles with high positive surface charges can aggregate

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unfavourably[98]. Moreover, adsorption of serum opsonins can tag particles for uptake by the mononuclear phagocyte system[70], a common mechanism through which delivery systems are cleared from the circulation and prevented from reaching target tissues. An accepted strategy for minimizing nonspecific interaction with serum components and thereby increasing circulation time involves steric shielding of the surface of delivery vehicles with hydrophilic polymers (often PEG)[70,71].

A major route of elimination of siRNA from the bloodstream is through kidney filtration. As naked siRNA can pass through the glomerular filtration barrier[99], many delivery systems prevent renal clearance by encapsulating siRNA into particles larger than ~20 nm[17]. It should be noted that several highly efficacious delivery systems, including Dynamic PolyConjugates (DPCs)[100] and triantennary *N*-acetylgalactosamine (GalNAc) conjugates[101], are smaller than this cutoff, which probably reflects the fact that these delivery systems mainly deliver their payloads in the first pass through the liver. Some nanoparticulate systems undergo disassembly in the glomerulus[102,103]. Interaction with the negatively charged glomerular basement membrane can disrupt the electrostatic interactions that are essential to the stability of certain particles, which causes disassembly and passage of siRNA into urine[102,103].

Delivery systems that are not eliminated by degradation, phagocytosis or glomerular filtration can leave the bloodstream by crossing the endothelium to reach target tissues. This occurs most readily in tissues with discontinuous endothelia, such as in liver tissues and many solid tumours[90]. Fenestrations in the liver sinusoidal endothelium permit particles of 100–200 nm in diameter to exit the bloodstream and gain access to hepatocytes and other liver cells[104,105]. In some tumours, a combination of highly permeable endothelia and poor lymphatic drainage can lead to increased accumulation of circulating nanoparticles in malignant tissue — an occurrence termed the enhanced permeation and retention effect[106].

Cellular uptake of siRNA delivery nanoparticles most often occurs through endocytosis. To enhance the rate of cell entry, many delivery systems incorporate ligands that bind specifically to receptors on target cells to trigger receptor-mediated endocytosis[107]. This ligand–receptor interaction can be hindered by serum proteins adsorbed on the surface of the delivery vehicle[108]. Endocytosed materials are entrapped in membrane-bound endocytic vesicles, which fuse with early endosomes and become increasingly acidic as they mature into late endosomes. Some delivery materials respond to the low pH environment in endosomes by becoming membrane-destabilizing, thereby enabling siRNA to escape from endosomes into the cytoplasm[14,100]. For many delivery systems, the precise mechanism of endosomal release is poorly understood. The exact intracellular trafficking pathways that affect delivery are also unclear. A recent report showed that for a leading lipid delivery system, ~70% of the siRNA taken up by cells underwent endocytic recycling and exocytosis[109]. An improved understanding of both endosomal release and intracellular trafficking may facilitate the design of delivery systems.

In the cytosol, siRNA must be loaded into the RNA-induced silencing complex (RISC) to activate the RNAi pathway. The siRNA strand with the least stably hybridized 5′ terminus becomes preferentially loaded into the RISC as the guide strand, and the other strand is cleaved. Covalent attachment of delivery materials to the 5′ end of the guide strand is typically avoided because this end is essential for RISC loading[110]. The siRNA sequence and backbone modifications must be selected to ensure appropriate strand selection by RISC and to avoid partial hybridization to non-target mRNAs, which can induce off-target gene silencing[90].

Lipid-based siRNA nanoparticles. One class of lipid-based siRNA vectors under clinical evaluation is termed the stable nucleic acid–lipid particle (SNALP) formulation[14,111,112] (FIG. 3a). The SNALP formulation was adapted from a protocol for DNA delivery and involves encapsulating nucleic acids into lipid-based nanoparticles (LNPs) that are <200 nm in diameter[113]. The first SNALP formulation for siRNA delivery was reported in 2005 and targets the hepatitis B virus (HBV) in a mouse model for HBV replication[114]. Most SNALP formulations can effectively deliver nucleic acids into hepatocytes, and all of the SNALP-targeted genes in clinical trials are disease-relevant targets in the liver. The *in vivo* mechanisms of siRNA delivery by some SNALP formulations involve apolipoprotein E (APOE)-dependent internalization of these particles into hepatocytes[97]. Interestingly, the LNP–APOE association has been reported to be dependent on pK_a, and more weakly basic cationic lipids ($pK_a \sim 6.5$) show higher APOE dependence[115]. However, studies have identified lipid components with higher pK_a that can also facilitate delivery to hepatocytes in an APOE-independent manner[115,116]. A recent study reports that the pK_a of a LNP, rather than the pK_a of individual lipids, is a key determinant of LNP activity *in vivo*[117]. Structurally novel lipid components for LNP formulations have been identified with a range of *in vivo* delivery competence[14,15], and understanding their underlying mechanisms will further aid our knowledge of the properties that determine LNP-mediated delivery.

Several SNALP-based formulations have been studied in clinical trials[118] (TABLE 2). For treatment of hypercholesterolemia, PRO-040201 (TKM-ApoB; Tekmira Pharmaceuticals Corporation), which targets APOB production, and ALN-PCS02 (Alnylam Pharmaceuticals), which targets the proprotein convertase subtilisin/kexin type 9 (*PCSK9*) transcript[118,119], have been reported to substantially reduce expression of their target genes and subsequently of low-density lipoprotein (LDL) cholesterol in Phase I trials. Whereas the TKM-ApoB trial was terminated owing to siRNA-dependent immunogenicity, the ALN-PCS02 trial has been continued with no serious adverse effects reported[119]. Two separate clinical trials are assessing the effects of LNP-based siRNA delivery in patients with hepatocellular carcinoma: TKM-080301 (Tekmira Pharmaceuticals Corporation) is being evaluated as an antitumour drug that suppresses polo-like kinase 1 (PLK1), and ALN-VSP02 (Alnylam Pharmaceuticals) is being developed to simultaneously

RNAiMAX
A commercial reagent that delivers small interfering RNAs into various types of cells *in vitro* with high efficiency. As a cationic lipid formulation, it can also be used to deliver microRNA antagonists and mimics, as well as mRNAs.

Glomerular filtration barrier
A blood filtration interface in the kidney that allows free passage of small ions such as sodium and potassium but that retains large proteins. The cutoff to pass this barrier is ~70 kDa, and naked small interfering RNAs (~13 kDa) can thus be filtered through the kidney.

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a

b

c

PBAVE

GalNAc and PEG released in the endosome

siRNA released in the cytoplasm

d

DSPC

DLinDMA

PEG-C-DMA

AD–PEG

AD–PEG–Tf

Cholesterol

CDP

siRNA

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◀ Figure 3 | **Structure of non-viral siRNA vectors. a** | A stable nucleic acid–lipid particle (SNALP) is shown. The composition of the SNALP formulation is generally a mixture of the 'helper lipid' DSPC (yellow), cholesterol (orange), a cationic lipid such as DLinDMA (blue), and a polyethylene glycol (PEG)–lipid known as PEG-C-DMA. These components form particles with a lipid bilayer structure, and the chemical structures of these components are shown in the box. The PEG–lipids provide a neutral hydrophilic exterior that enhances particle stability and minimizes clearance[152]. After administration, the PEG–lipid component dissociates from the particle at a defined rate, thereby exposing the cationic lipid component of the formulation, which is crucial for cell transfection[153]. The cationic lipid component has a key role in cellular internalization of the particles, whereas the helper lipid component is important for escape of the payload from the endosomal compartment. **b** | A Cyclodextrin polymer (CDP)-based nanoparticle is shown. CDPs are synthesized through polymerization of diamine-bearing cyclodextrin (dark green) and dimethyl suberimidate, which yields oligomers ($n \sim 5$) with amidine groups (light blue)[35]. The positively charged amidine groups interact with nucleic acids to form stable particles. The polymers are end-capped with imidazole functional groups, which have been shown to facilitate endosomal escape[154] and improve delivery efficacy of small interfering RNAs (siRNAs)[125]. Adamantane (AD) is a hydrophobic molecule that forms a stable inclusion complex with the cyclic core of cyclodextrin[155]. CDP–siRNA nanoparticles are formulated with PEG (the molecular mass of which is 5,000) for stability and long circulation time, as well as with transferrin (Tf), which induces the uptake by cells expressing the transferrin receptor[155,156]. **c** | A Dynamic PolyConjugate (DPC) is shown. DPCs are composed of a membrane-destabilizing polymer PBAVE, the activity of which is shielded by PEG during circulation. *N*-acetylgalactosamine (GalNAc) moieties bind to receptors on hepatocytes to induce uptake of the vector by endocytosis. In the acidic environment of the endosome, PEG and GalNAc moieties are released, which exposes the membrane-disrupting polymer to promote endosomal release. The siRNA is conjugated to the polymer through a hydrolysable disulphide linker, which is reduced in the cytosol to release the siRNA[100]. **d** | A GalNAc–siRNA conjugate is shown. The chemically stabilized siRNA is conjugated at the 3′ terminus of the passenger strand to three GalNAc molecules through a triantennary spacer molecule. The spacer length, valency and ligand spacing are optimized for high-affinity binding to its receptor on hepatocytes[101]. Part **a** reprinted from *Curr. Opin. Pharmacol.*, Vol. 12 (4), Alabi, C. *et al.* Attacking the genome: emerging siRNA nanocarriers from concept to clinic. 427–433 © (2012), with permission from Elsevier. Parts **b**, **c** and **d** from REF. 101, Nature Publishing Group.

target *KIF11* (which encodes kinesin spindle protein) and *VEGF*. In addition to these indications, the siRNA formulation ALN-TTR02 (Alnylam Pharmaceuticals) is being evaluated for its ability to suppress mutant transthyretin (TTR) in TTR-mediated amyloidosis. The second-generation SNALP formulation ALN-TTR02 (also known as Patisiran) features a DLinDMA analogue that has showed a tenfold increase in efficacy in preclinical studies[115].

There are also two clinical trials that feature LNP formulations other than SNALP. Silence Therapeutics is evaluating their LNP system, AtuPLEX, which consists of a cationic lipid (AtuFECT01), a helper lipid (DPhyPE) and a PEG–lipid (PEG–DSPE) in a 50:49:1 ratio with siRNAs[120]. This formulation was shown to internalize into mouse vascular endothelium after intravenous injection[120,121]. The AtuPLEX-based formulation Atu027 features an siRNA that targets the protein kinase N3 (*PKN3*) transcript and is under evaluation for the treatment of patients with advanced solid cancer[122] (TABLE 2). Finally, the MD Anderson Cancer Center has initiated a clinical trial for their formulation of siRNA–EphA2–DOPC, in which siRNA is incorporated into neutral liposomes composed of DOPC[123]. This siRNA targets *EPHA2* (which encodes a tyrosine kinase) and is being evaluated in patients with advanced cancers (TABLE 2).

Polymer-based siRNA nanoparticles. Cyclodextrin polymer (CDP)-based nanoparticles (FIG. 3b) were the first targeted nanoparticulate siRNA delivery system to enter clinical trials for cancer[12]. The cyclodextrin delivery system was first introduced to deliver plasmid DNA in 1999 (REF. 13), and the system was re-optimized for siRNA delivery years later[124,125]. CDPs are of interest as delivery polymers owing to their low toxicity and polycationic charge. The CDP–siRNA delivery system has been evaluated in several therapeutically relevant animal models and in Phase I clinical trials. This system was reported to silence cancer-associated gene targets in mouse models[125,126]. Its clinical translatability was evaluated in cynomolgus monkeys, in which the CDP–siRNA delivery system indicated efficacy in the range of 0.6–1.2 mg siRNA per kg (body weight) and tolerability up to 27 mg siRNA per kg (body weight)[127]. Finally, the clinical potential of CDP-based RNAi delivery was established in a Phase I clinical trial (TABLE 2), in which the ribonucleotide reductase M2 (*RRM2*) mRNA was targeted in patients with solid cancers. A reduction in target mRNA levels was reported, and the presence of the specific mRNA cleavage product supports an RNAi mechanism of action[128].

Conjugate siRNA delivery systems. Several promising delivery systems have been developed through covalent attachment of delivery ligands to the siRNA cargo, which yields precisely defined, single-component systems that require minimal delivery material. The most clinically advanced conjugate platforms are DPCs and GalNAc conjugates. These two systems are proprietary technologies and therefore have not been frequently described in the scientific literature. Nonetheless, these platforms provide informative lessons to guide the development of conjugate delivery systems.

DPCs were originally developed as a composition of siRNA conjugated to polymers and ligands in a formulation that is designed to respond to intracellular environments to trigger the release of siRNA. The system includes a membrane-disrupting polymer, shielding polymers and targeting ligands, and each component is designed to provide a particular function in the delivery process (FIG. 3c).

DPCs aimed at treating liver diseases are targeted to hepatocytes using GalNAc ligands, which bind to the asialoglycoprotein receptor. When administered intravenously in mice, first-generation DPCs effectively silenced endogenous liver genes, including *ApoB*, in a dose-dependent manner and produced the expected phenotypic effects[100]. Newer generations of DPCs under development by Arrowhead Research Corporation aim to expand on this original platform to improve performance. To target organs other than the liver, the company reports the use of different targeting ligands and the more stable attachment of the PEG shielding agent to increase circulation time. According to the company's website, the latest generation of DPCs induced 99% silencing of liver genes in non-human primates after a single dose of 0.2 mg DPC per kg (body weight), and the effect lasted nearly 7 weeks (see Dynamic

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Table 2 | **Non-viral siRNA vectors under clinical evaluation**

Delivery system	Drug	Sponsor	Target gene	Disease	Phase	Status	ClinicalTrials.gov identifier
Naked siRNA	ALN-RSV01	Alnylam Pharmaceuticals	Nucleocapsid gene of RSV	RSV infections	II	Completed	NCT00658086
	TD101	Pachyonychia Congenita Project	*KRT6A* (N171K mutation)	Pachyonychia congenita	I	Completed	NCT00716014
	AGN211745	Allergan	*FLT1*	Age-related macular degeneration and choroidal neovascularization	II	Terminated	NCT00363714
	QPI-1007	Quark Pharmaceuticals	*CASP2*	Optic atrophy and non-arteritic anterior ischemic optic neuropathy	I	Completed	NCT01064505
				Acute primary angle-closure glaucoma	II	Active	NCT01965106
	I5NP	Quark Pharmaceuticals	*TP53*	Kidney injury and acute renal failure	I	Completed	NCT00554359
				Delayed graft function and complications of kidney transplant	I/II	Active	NCT00802347
	PF-655 (PF-04523655)	Quark Pharmaceuticals	*DDIT4*	Choroidal neovascularization, diabetic retinopathy and diabetic macular edema	II	Completed	NCT01445899
				Age-related macular degeneration	II	Completed	NCT00713518
	Bevasiranib	OPKO Health, Inc.	*VEGFA*	Diabetic macular edema	II	Completed	NCT00306904
				Macular degeneration	II	Completed	NCT00259753
	SYL1001	Sylentis S.A.	*TRPV1*	Ocular pain and dry eye syndrome	I/II	Recruiting	NCT01776658
	SYL040012	Sylentis S.A.	*ADRB2*	Ocular hypertension and open angle glaucoma	II	Completed	NCT01739244
	RXI-109	RXi Pharmaceuticals	*CTGF*	Cicatrix and scar prevention	I	Active	NCT01780077
				Hypertrophic scar	II	Recruiting	NCT02030275
				Keloid	II	Recruiting	NCT02079168
Lipid-based nanoparticles	ALN-VSP02	Alnylam Pharmaceuticals	*KIF11* and *VEGF*	Solid tumours	I	Completed	NCT01158079
	siRNA–EphA2–DOPC	MD Anderson Cancer Center	*EPHA2*	Advanced cancers	I	Active	NCT01591356
	Atu027	Silence Therapeutics	*PKN3*	Advanced solid cancers	I/II	Recruiting	NCT01808638
	TKM-080301	Tekmira Pharmaceuticals Corporation	*PLK1*	Cancer	I/II	Recruiting	NCT01262235
	TKM-100201	Tekmira Pharmaceuticals Corporation	*VP24*, *VP35* and Zaire Ebola L-polymerase gene	Ebola virus Infection	I	Terminated	NCT01518881
	PRO-040201	Tekmira Pharmaceuticals Corporation	*APOB*	Hypercholesterolemia	I	Terminated	NCT00927459
	ALN-PCS02	Alnylam Pharmaceuticals	*PCSK9*	Hypercholesterolemia	I	Completed	NCT01437059
	ALN-TTR02	Alnylam Pharmaceuticals	*TTR*	TTR-mediated amyloidosis	III	Recruiting	NCT01960348
	ND-L02-s0201	Nitto Denko Corporation	*SERPINH1*	Fibrosis	I	Completed	NCT01858935
CDP-based nanoparticle	CALAA-01	Calando Pharmaceuticals	*RRM2*	Solid tumours	I	Terminated	NCT00689065
Dynamic Poly-Conjugate	ARC-520	Arrowhead Research Corporation	Two conserved regions of HBV transcripts	Hepatitis B	I	Recruiting	NCT01872065
					II	Recruiting	NCT02065336

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Table 2 (cont.) | **Non-viral siRNA vectors under clinical evaluation**

Delivery system	Drug	Sponsor	Target gene	Disease	Phase	Status	ClinicalTrials.gov identifier
siRNA–GalNAc conjugate	ALN-TTRsc	Alnylam Pharmaceuticals	*TTR*	TTR-mediated amyloidosis	I	Recruiting	NCT01814839
					II	Recruiting	NCT01981837
LODER polymer	siG12D LODER	Silenseed Ltd.	*KRAS*	Pancreatic cancer	II	Active	NCT01676259

ADRB2, adrenoceptor beta 2, surface; *APOB*, apolipoprotein B; *CASP2*, caspase 2, apoptosis-related cysteine peptidase; CDP, cyclodextrin polymer; *CTGF*, connective tissue growth factor; *DDIT4*, DNA-damage-inducible transcript 4 (also known as *RTP801*); *EPHA2*, EPH receptor A2; *FLT1*, fms-related tyrosine kinase 1 (also known as *VEGFR1*); GalNAc, *N*-acetylgalactosamine; HBV, hepatitis B virus; *KIF11*, kinesin family member 11; *KRT6A*, keratin 6A; *PCSK9*, proprotein convertase subtilisin/kexin type 9; *PKN3*, protein kinase N3; *PLK1*, polo-like kinase 1; *RRM2*, ribonucleotide reductase M2; RSV, respiratory syncytial virus; *SERPINH1*, serpin peptidase inhibitor, clade H, member 1 (also known as *HSP47*); siRNA, small interfering RNA, *TP53* encodes p53; *TRPV1*, transient receptor potential cation channel, subfamily V, member 1; *TTR*, transthyretin; *VEGFA*, vascular endothelial growth factor A.

PolyConjugates (DPC) technology: an elegant solution to the siRNA delivery problem).

Among the new generation DPCs that target the liver are DPCs in which the masked PBAVE polymer is not covalently attached to the siRNA cargo but is instead co-injected with cholesterol-modified siRNA[129]. Although the two components did not interact in solution, they were both targeted to hepatocytes and colocalized in endosomes, even when they were injected separately. The cholesterol moiety, the asialoglycoprotein receptor and the GalNAc ligand were all required for silencing activity. Interestingly, LDL and the LDL receptor were not essential for gene silencing, although they were required for silencing by cholesterol–siRNA alone[96]. Arrowhead Research Corporation's clinical candidate ARC-520 uses this co-injection strategy to target two conserved regions of HBV transcripts. The drug contains a reversibly masked melittin-like peptide in place of the original PBAVE polymer and two cholesterol-modified siRNAs[130,131]. ARC-520 is currently in Phase I clinical trials (TABLE 2).

A leading conjugate delivery platform that is currently under development at Alnylam Pharmaceuticals, siRNA–GalNAc conjugates, contains a highly modified siRNA that is stably conjugated to a multivalent targeting ligand (FIG. 3d). This platform is a component of several drug candidates of the company, including ALN-TTRsc, ALN-PCS, ALN-AT3 and ALN-AS1 (REF. 101). ALN-TTRsc, which is designed to silence *TTR* for the treatment of TTR-mediated amyloidosis, is the most clinically advanced of all siRNA–GalNAc conjugates developed by the company so far. In a Phase I clinical trial, ALN-TTRsc could stably reduce serum TTR levels by >90% with regular subcutaneous administration at a dose of 10 mg per kg (body weight) and by >50% after a single dose of 10 mg per kg (body weight) (see ALN–TTRsc Phase I study results) (TABLE 2). Serum TTR levels gradually returned to pre-treatment levels upon cessation of treatment. The drug was generally safe and well tolerated, and Phase II clinical studies began at the end of 2013. Three other drug candidates (ALN-PCS, ALN-AT3 and ALN-AS1 (REF. 132)) have the same GalNAc conjugate platform but different siRNA sequences that target the expression of various hepatocellular proteins (PCSK9, antithrombin and aminolevulinate synthase 1, respectively). These drugs have shown efficacy in animal models and are now under development for treating hypercholesterolemia, haemophilia and hepatic porphyrias, respectively[101].

In summary, among the siRNA delivery systems that have shown efficacy in animals, there is much diversity in the size, shape, chemical properties, structure and method of development. Synthetic LNP formulations are currently among the most potent delivery systems. Conjugates benefit from well-defined structures with minimal delivery material and apparently broad therapeutic windows, and there is continued progress in improving their potency. There are many physiological considerations to address in designing materials to deliver siRNAs to target cells, and parts of the delivery process remain poorly understood. The continued study of new and diverse delivery strategies will help to elucidate the underlying biological processes that influence delivery and will guide the design of future generations of siRNA delivery platforms.

In vivo delivery of miRNA mimics

Since miRNAs were discovered 20 years ago, their functions have been intensively investigated and their mechanisms are well studied. Often 18–25 nucleotides in length, miRNAs are endogenously synthesized and promote mRNA degradation and/or translational inhibition[133]. It has been reported that the human genome contains >1,000 miRNAs, and each of them might regulate hundreds of genes under certain conditions[134]. Whereas more than a dozen of clinical trials assess miRNAs as biomarkers, the clinical use of the most potent miRNAs as therapeutics remains challenging.

Levels of miRNAs can be reduced by inhibitory drugs referred to as antagomirs, which are usually short single-stranded oligonucleotides with locked nucleic acid modifications. Owing to the size, charge and stability of antagomirs, endogenous proteins can potentially be used as carriers. At a fairly large dose, antagomirs can even be delivered *in vivo* without exogenous delivery vehicles. Thus, we do not discuss clinical trials using antagomirs, which have recently been reviewed elsewhere[135].

Levels of miRNAs can be restored through the introduction of synthetic miRNAs or miRNA mimics, and this approach is referred to as miRNA replacement therapy. Similarly to therapeutic siRNAs, miRNA replacement therapy requires delivery carriers. As synthetic miRNA

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*Fok*I
A restriction endonuclease with a DNA-binding domain at the amino terminus and a nonspecific DNA cleavage domain at the carboxyl terminus. Dimerization of two *Fok*I is required to cleave DNA. When it is fused to zinc-finger proteins (ZFPs) or to transcription activator-like effectors (TALEs), *Fok*I will function when a pair of ZFPs or TALEs binds to DNA within short distance. This strategy allows increased specificity of sequence recognition.

is similar to siRNA in terms of structure, size and charge, the delivery barrier of miRNA could be the same as that of siRNA, and it is likely that systems for siRNA delivery can also be used for miRNA delivery in clinical trials.

Several candidates are under preclinical evaluation, mostly for oncology studies owing to the ability of miRNAs to downregulate multiple cancer-relevant genes[136]. MRX34 (Mirna Therapeutics), which is a miRNA-34a (miR-34a) mimic, is currently in a Phase I clinical trial (NCT01829971) of patients with primary liver cancer or liver metastases and is expected to finish in December 2014. miR-34a is a component in the p53 signalling pathway and a well-investigated tumour suppressor[137]. miR-34a replacement has shown potent antitumour activity *in vitro* and *in vivo*[137,138]. According to Mirna Therapeutics, the formulation used in the trial is based on an ionizable liposome with a diameter of ~120 nm[139]. The nanoparticles contain amphoteric lipids that are neutral or anionic at high pH and cationic at low pH[139]. The detailed structure of this liposome has not yet been revealed.

Although there are >1,000 miRNAs, most of them have a role in 'fine-tuning' gene expression, and only a small percentage of miRNAs show potent functionality across multiple targets. Nevertheless, off-target effects are of concern. However, potential off-target sites can be predicted by bioinformatic analyses, and the level of inhibition of each target can vary. By contrast, the off-target effects of small-molecule inhibitors are hard to predict. In fact, many small-molecule drugs can bind to numerous different targets, usually in a dose-dependent manner. Currently, miRNA therapeutics is explored for severe diseases such as cancer.

Delivery of genome editing systems

Conventional gene therapy either temporarily introduces expression of a DNA fragment or randomly inserts it into the genome. A more precise way to repair disease-causing genes is needed. The sequence-specific ZFPs, TALEs and CRISPR–Cas systems not only have great potential to revolutionize biomedical research as powerful tools but also provide feasible platforms for personalized medicine[140,141]. ZFPs and TALEs can target unique sequences in the genome following customization of their DNA-binding domains[141–143]. Fusion of ZFPs and TALEs with a DNA cleavage module, such as the cleavage domain of the *Fok*I endonuclease, creates chimeric zinc-finger nucleases (ZFNs) and TALE nucleases (TALENs)[140,144] (FIG. 4). Distinct from ZFNs and

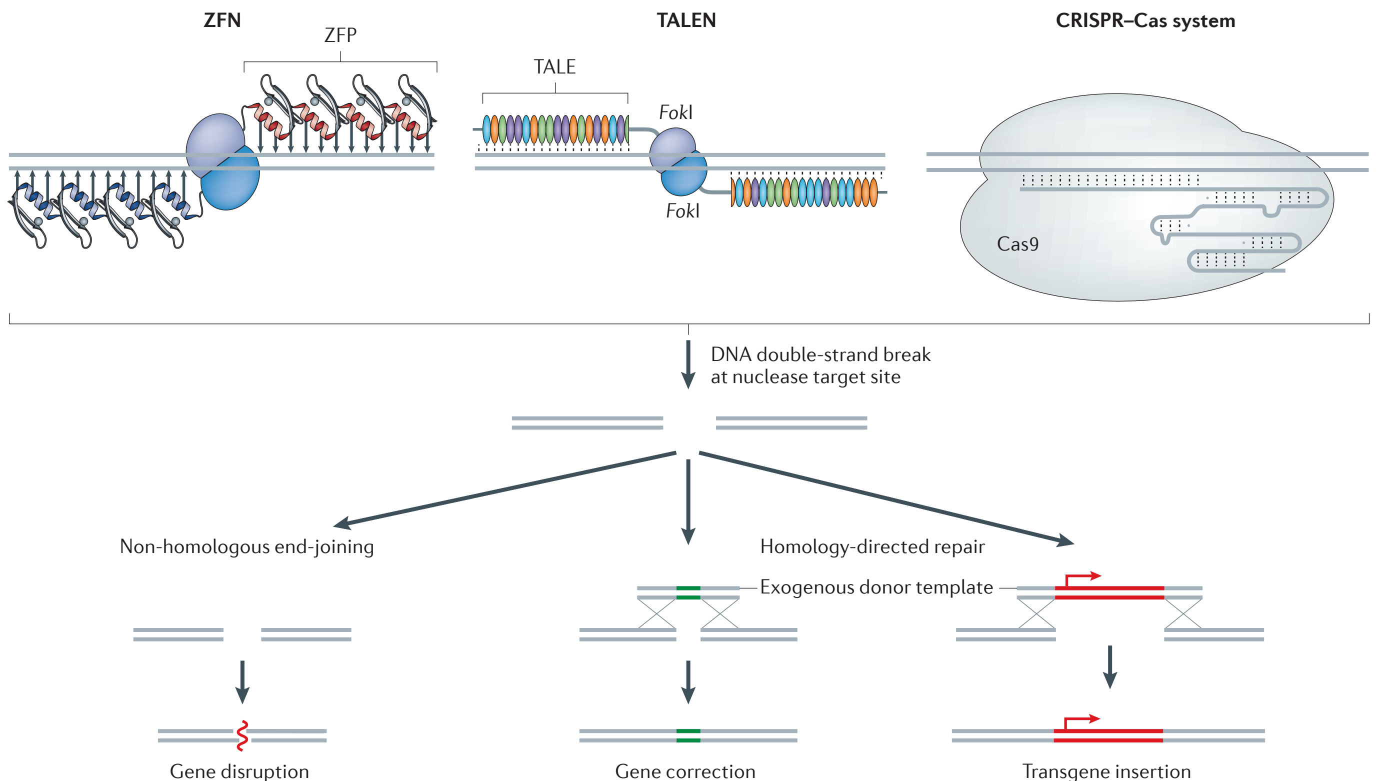

Figure 4 | **Potential use of non-viral vectors for the delivery of precise genome editing systems.** The mRNA or DNA encoding a zinc-finger nuclease (ZFN)[157], a transcription activator-like effector nuclease (TALEN) or CRISPR–Cas (clustered regularly interspaced short palindromic repeat–CRISPR-associated) systems and a repair template could potentially be introduced in complex with non-viral vectors. These engineered nucleases can manipulate mammalian genomes with precision and high efficiency by creating double-strand breaks (DSBs) or nicks in one strand in targeted genomic sequences[140]. The DSB is repaired by non-homologous end-joining or by homology-directed repair if donor templates are provided. Efficient genome engineering using ZFN, TALEN and CRISPR–Cas systems allows precise correction of disease-causing genes. With successful delivery of these genome editing systems, there is potential for one or more disease-causing genes to be precisely corrected in a certain number of cells *in situ*, which allows cure of diseases with minimal adverse effects. ZFP, zinc-finger protein.

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TALENs, the DNA endonuclease Cas9 (which is derived from the bacterial CRISPR–Cas system) can recognize and induce DNA double-strand breaks in a genomic site through a synthetic guide RNA that hybridizes with the target DNA sequence[145,146] (FIG. 4). RNA-guided Cas9 enables faster and easier implementation than ZFNs and TALENs, and it has great potential to simultaneously engineer multiple sites in the genome[145–147]. There are ongoing efforts to increase the efficiency of CRISPR–Cas systems and to decrease their off-target effects[148].

Ongoing clinical trials (NCT01044654, NCT01252641 and NCT00842634) use ZFNs to disrupt the C-C chemokine receptor type 5 (CCR5)-encoding gene in $CD4^+$ T cells *ex vivo* to promote HIV-1 resistance. *In vivo* gene correction by ZFN was achieved using AAV as the delivery system in a mouse model of haemophilia[149]. We showed that CRISPR–Cas9 corrected a disease mutation of hereditary tyrosinaemia in adult mice and rescued the disease phenotype[150].

High efficiency of these programmable genome modification systems provides the possibility of precise *in vivo* gene editing using non-viral delivery vectors. In principle, non-viral vectors that carry genome editing systems are highly appealing platforms to permanently correct disease mutations, such as point mutations and deletions, without any involvement of viruses, thereby minimizing the chance of short-term and long-term adverse effects. Considering the current limitations of non-viral delivery vectors, likely target tissues for such precise genome editing systems include hepatocytes, endothelial cells and epidermal cells.

Conclusions

In the past decades, substantial advances have been achieved in different areas related to gene-based therapy, including the development of new delivery materials, as well as improved potency and stability of nucleic acids. Moreover, advances in genomics have greatly enhanced our understanding of the genetic basis of disease and provided a range of new targets for genetic medicine. Many recently identified disease targets are considered 'undruggable' using small-molecule inhibitors[151]. The potential of exogenous nucleic acids may provide an avenue to translate our knowledge obtained from biomedical research into therapeutics. The broad application of non-viral gene-based therapy will require continued efforts not only to understand the structure–function relationship and the biology behind delivery vectors but also to expand the list of tissues and organs that can be successfully targeted. In addition, non-viral delivery of genome editing systems could facilitate both precise and permanent correction of disease genes. The development of advanced delivery vectors should increase the potential of gene-based therapy to treat a range of different tissues and to silence, correct or introduce specific genes with minimal adverse effects.

More than a decade after the initial discovery of RNAi, the promise of RNAi-based drugs seems imminent. Several therapeutic approaches based on siRNA have shown promising results in clinical trials and have illuminated the challenges associated with the application of RNAi in humans[101]. With the recent development of nanoparticle and conjugate formulations, *in vivo* delivery of siRNAs and/or miRNAs can be achieved with high efficiency and low toxicity, and efficacy has been shown in humans. Although non-viral gene-based therapies have yet to be approved by the FDA, recent progress in the clinical development of RNAi has generated considerable excitement. As discussed, several clinical programmes have reported targeted knockdown in humans following non-viral delivery of siRNAs[101,119]. One compound, ALN-TTR02, has now transitioned into Phase III clinical trials. More recently, modified siRNAs conjugated to liver-targeting ligands have shown efficacy in humans following subcutaneous application, which highlights the possibility of siRNA therapeutics that can be self-administered by patients (see ALN–TTRsc Phase I study results). Taken together, although it will be important to monitor performance of these drugs as they advance into later-stage trials, the efficacy already shown by these formulations in humans, together with the many advances in preclinical development, convinces us that siRNA has a broad and important potential as therapeutics in humans.

Whereas the promise of RNAi-based therapies seems to be within reach, there are still certain challenges for successful mRNA- and DNA-based therapies using non-viral vectors. The challenges of stability are greater for mRNA than for siRNA, as the chemical modifications that confer stability on siRNA duplexes can often render mRNAs ineffective. Therapeutic DNA expression additionally requires delivery into the nucleus, which still poses a formidable challenge. Nonetheless, the immense therapeutic potential of these technologies is on the horizon, and the clinical translation of siRNA delivery systems will help to guide us in overcoming these additional barriers.

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Competing interests statement

The authors declare competing interests: see Web version for details.

FURTHER INFORMATION

ALN–TTRsc Phase I study results: http://www.alnylam.com/web/wp-content/uploads/2013/09/ALN-TTRsc-PhaseI-Results-HFSA-Sept2013.pdf

Alnylam Pharmaceuticals: www.alnylam.com

Dynamic PolyConjugates (DPC) technology: an elegant solution to the siRNA delivery problem: http://www.arrowheadresearch.com/sites/default/files/udocs/Arrowhead_Research_Corporation-DPC_Technology_White_Paper.pdf

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